RESEARCH ARTICLE

The inhibitory effect of baicalin on viral diarrhea and mucosal disease in livestock and cattle

Jun Wu*, Erxue Xu

Department of Biology and Health, Chizhou Vocational and Technical College, Chizhou, Anhui, China.

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Bovine viral diarrhea virus is a type of envelope virus that can cause bovine viral diarrhea with the symptoms of gastrointestinal mucosal ulcers, gastrointestinal inflammation, and diarrhea. The presence of viral infection is usually identified through antigen and polymerase chain reaction (PCR) testing. Baicalin is a flavonoid extracted from the dried root of *Scutellaria baicalensis Georgi*, which has shown unique advantages in the treatment of viral infectious diseases. This study analyzed the activity and mechanism of baicalin to explore its antiviral effect on bovine viral diarrhea virus using a control experiment method to divide Mada's bovine kidney cells into normal cells, virus control, and experimental groups. The infection dose and OD_{450} value of half of the cell tissue culture were measured, and the effect of baicalin on autophagy gene expression was detected using fluorescence dye method. The results showed that cells infected with bovine viral diarrhea virus had blurred boundaries and exhibited pathological changes. As the concentration of baicalin gradually increased, the OD_{450} value, cell survival rate, and antiviral efficacy also gradually increased. When the concentration of baicalin was 12.5 μg/mL, the OD_{450} was 0.714, the cell survival rate was 89.06%, and the antiviral efficiency of baicalin was 85.83%. The IC_{50} of baicalin on bovine kidney cells was 45.31 μg/mL. The copy number of deoxyribonucleic acid in the combination of baicalin and bovine viral diarrhea virus group was significantly lower than that in the bovine viral diarrhea virus group. The group with bovine viral diarrhea virus had the most cell apoptosis, while the group with baicalin combined with bovine viral diarrhea virus had the least cell apoptosis. The results demonstrated the good antiviral ability, invasion blocking effect, and inhibitory effect of baicalin on bovine viral diarrhea virus. This study can provide some references for the development of vaccines and antiviral drugs for the prevention of bovine viral diarrhea virus infection.

Keywords: bovine viral diarrhea mucosal disease; baicalin drugs; antiviral drugs; intrusion blocking.

***Corresponding author:** Jun Wu, Department of Biology and Health, Chizhou Vocational and Technical College, Chizhou 247100, Anhui, China. Email: maggie3321123@163.com.

Introduction

The rapid progress of the economy and the continuous increase in market demand have led to the rapid development of agriculture and animal husbandry. The health of agricultural and livestock products is also receiving increasing attention. Bovine viral diarrhea mucosal disease (BVD-MD) is a contagious disease caused by bovine viral diarrhea virus (BVDV). Cattle of all ages are susceptible to BVDV with young cattle being the most susceptible one, which has a serious impact on food safety [1, 2]. In addition to BVD-MD, BVDV can also cause the occurrence of various diseases, and there have been cases of infected individuals. Therefore, it is crucial to

effectively suppress BVDV, which is related to economic development and human health [3]. BVD-MD has also caused huge economic losses to animal husbandry, posing a serious threat to the development of China's animal husbandry industry. However, due to the complex pathogenic mechanism of BVDV, there is currently no specific preventive vaccine or antiviral drug for BVDV infection. As researchers increasingly focus on minimizing side effects and developing non-resistant drugs, the development of traditional Chinese veterinary drugs for treating viral diseases has emerged as a significant research area [4].

BVD-MD is mainly characterized by fever, mucosal erosion and necrosis, diarrhea, thrombocytopenia and leukopenia, miscarriage, and abnormal fetuses. Chi *et al*. stated that BVDV had a serious negative impact on the development of the global livestock industry and reviewed the functions of BVDV's non-structural proteins, which helped to gain a more comprehensive understanding of the virus replication process and the molecular mechanisms of persistent virus infection. The results provided a certain reference for the diagnosis and prevention of BVD-MD, identification of BVDV, and vaccine development [6]. Kučer *et al*. conducted genetic analysis on the types of BVDV to confirm the presence of BVDV in dairy herds using serum samples from clinically infected cows and tested for the presence of BVDV antibodies and antigens. The virus neutralization test showed that 194 out of 233 tested cows tested positive, confirming the BVDV-1d sub-type for the first time in Croatia [7]. The control of BVD-MD by farmers may be influenced by psychological and social factors, but many of these factors have not been studied in the context of farmer control of infectious diseases. Prosser *et al*. investigated the psychological and social status of farmers using various effective measures to address this issue. The control of BVD-MD by farmers was related to the national strategy for controlling BVD-MD [8]. Wernike *et al*. studied the ring test sample group using nine commercially available antibodies and neutralization tests targeting different BVDV-1, BVDV-2, and border disease virus strains. Although serum negative samples had been correctly evaluated in most cases, there were significant differences in the number of correctly evaluated samples, especially when testing mixed milk samples [9]. Hashemi *et al*. randomly selected serum samples from 1,036 cows for virus antibody testing to determine the serum prevalence of BVDV, bovine herpesvirus α type 1, and bovine parainfluenza virus type 3 (BPVT-3) at both population and animal levels. BVDV and BPVT-3 infections were common in cattle in northwestern Iran, and this study was beneficial for relevant departments to take targeted prevention and control measures [10]. Zhang *et al*. observed the histopathological changes in the duodenum and spleen of mice before and after the addition of root bark glycoside to investigate the effect and mechanism of root bark glycoside on BVDV infection in the laboratory. The results suggested that ginsenoside had an inhibitory effect on BVDV and could significantly inhibit the replication of BVDV in mice [11].

Baicalin is a flavonoid compound extracted and isolated from the dry roots of *Scutellaria baicalensis* in the family of *Lamiaceae*, which has shown unique advantages in the treatment of viral infectious diseases in recent years. Numerous studies have demonstrated its significant biological activity and strong physiological efficacy in anti-cancer response. It also has functions such as purging fire, detoxifying, hemostasis, antibacterial, anti-inflammatory, anti-allergic, cholesterol lowering, anti-thrombotic, and relieving spasms [5]. Bao *et al*. described the current pharmacological effects of baicalin in regulating inflammation, oxidative stress, antiviral, and anti-tumor responses. The application of baicalin in livestock health and performance, biological activity, molecular mechanism, and dosage form were emphasized. The results confirmed that baicalin could improve the functions of various physiological systems and had strong anti-inflammatory effects [12]. Wang *et al*. provided an overview of the potential anticancer mechanisms of baicalin and discussed

the combination therapy strategy, clinical trials, and safety of baicalin as a chemotherapy adjuvant. Baicalin had good tolerance to cancer, but the deglycosylation of baicalin had stronger anti-cancer potential [13]. Adin *et al*. proposed a simple and accurate high-performance thin-layer chromatography method to verify the content of baicalin in different extracts. The results showed that ethanol extracts had significant antioxidant activity, and the content of baicalin was higher than that of acetone, dimethyl sulfoxide, and dimethylformamide extracts [14]. Wang *et al*. stated that using natural compounds as antidepressants had the advantages of fewer side effects and higher relief rates. In many preclinical studies conducted through various animal models of depression, baicalin demonstrated to have significant antidepressant activity. The molecular mechanism of baicalin's antidepressant effect was then reviewed [15]. Feng *et al*. discussed the *in vitro* anti-bronchitis virus activity and mechanism of action of baicalin in response to the unclear effect of baicalin on chicken infectious bronchitis virus. Baicalin had a direct antiviral effect on bronchitis virus infection, but its preventive effect was not significant [16]. Sharawi *et al*. stated that, in many studies, baicalin had been proven to protect the lungs in animal models, and therefore, analyzed data on the lung protective function of baicalin against various injuries. The anti-cancer activity with anti-cancer properties could effectively alleviate lung injury related to infection, and it had shown good effects on lung injury related to paraquat [17].

Many previous studies have been done extensively on BVDV and affirmed the anticancer, anti-inflammatory, and antiviral effects of baicalin. However, currently, there are relatively few studies on the treatment of BVD-MD with baicalin, and BVDV infection remains an important issue that needs to be urgently addressed. Existing antibiotics have significant side effects and are prone to causing pathogens to develop resistance. Natural compounds have attracted increasing attention from researchers due to their low toxicity, minimal side effects, and effectiveness [18]. Numerous studies have shown that flavonoids have significant antiviral effects. Baicalin is an indicator component of the natural medicine *Scutellaria baicalensis*, characterized by low toxicity, minimal side effects, and good biological activity. However, the water solubility and lipid solubility of baicalin are poor, resulting in low bioavailability, which limits its clinical application and pharmacological effects. This study investigated the activity and mechanism of baicalin against BVDV and explored the inhibitory effect of baicalin on BVD-MD using cytopathic effect (CPE) and cell counting kit-8 (CCK-8) methods. The results of this study would provide some references and basis for the development of traditional Chinese medicine preparations against BVDV, which would have a positive impact on the health care, production management, and economic benefits of animal husbandry.

Materials and methods

Resources of cells, baicalin, and BVDV

Madin Darby Bovine Kidney (MDBK) cells were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China). The cells were cultured using DMEM/High glucose medium (HyClone, Logan, Utah, USA) at 37℃ with 5% CO_2 in a D180 Carbon dioxide incubator (Ruiwode Life Technology Co., Ltd., Shenzhen, Guangdong, China) [19]. The baicalin stock solution was prepared by mixing 10 mg of baicalin (Pfizer Biotechnology Co., Ltd., Chengdu, Sichuan, China) with 1 mL of Dimethyl sulfoxide (DMSO) (Beijing Solebao Technology Co., Ltd., Beijing, China) to reach the concentration of 10 mg/mL. The stock solution was then diluted with cell culture medium to obtain the working concentrations of 0.049, 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, and 12.5 μg/mL. The BVDV NADL strain was obtained from China Institute of Veterinary Drug Control (Beijing, China) and was replicated by infecting MDBK cells and colleting virus containing supernatant of the culture.

Determination of Tissue Culture Infective Dose (TCID50)

TCID50 is an indicator used to measure the infectivity of viruses in cell culture systems and specifically refers to the amount of virus that can induce pathological effects in 50% of cultured cells after virus inoculation [20]. The BVDV solution was diluted 10 times with a dilution ratio of 10:10:10, and 8 replicates were set up for each dilution ratio. The mixed cell/BVDV cultures were put into a 5% CO_2 incubator at 37℃ for 5 days before measuring the virulence of BVDV using immunoperoxidase assay following the manufacturer's instructions. TCID50 of BVDV was determined by observing and recording the degree of cellular lesions and the number of pores under an Olympus IX73 Fluorescence Microscope (Olympus Corporation, Tokyo, Japan). The value of TCID50 for BVDV was calculated as:

$$\lg TCID_{50} = L - d(s - 0.5) \quad (1)$$

where L was the logarithm of the highest dilution of the virus. d was the difference between the logarithms of virus dilution. s was the total cell lesion pore rate.

The activity of baicalin against BVDV

To verify the invasion blocking effect of baicalin on BVDV, cells were divided into 3 groups with the normal cell group neither infected with BVDV nor treated with baicalin, the virus positive control group infected with BVDV only, and the experimental group infected with BVDV and treated with different concentrations of baicalin. Cells were added to a 96 well plate with 2.5×10^5 cells in each well. Upon growing into a single cell layer, 100 μL of baicalin in different working concentrations was added to each well and incubated for 4 h before removing the baicalin solution and adding 100 μL of 100 TCID50 BVDV virus solution. The cell culture contained BVDV was then incubated for 2 h with shaking thoroughly every 15 mins. After removing the virus solution, 200 μL of cultural medium was added to the cells. The cells were then put back to the 37℃, 5% CO_2 incubator for cultivation, and the pathological changes of the cells were observed every day. After five days of cultivation, 20 μL of Cell Counting Kit-8 (CCK-8) solution (Biyuntian Co., Ltd., Shanghai, China) was added to each well and incubated for 3 h before the OD_{450} value was measured using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, Shanghai, China). The cell survival rate and drug efficacy against BVDV in each group were then calculated.

The inhibitory effect of baicalin on BVDV

The sequences of BVDV primers and probes were designed based on the 5' UTR specific conserved region gene sequence of BVDV NADL strain using Primer 6.0 software [21] (Table 1). The primers and probes were synthesized by Shenggong Biotechnology Co., Ltd. (Shanghai, China). The MDBK cells were infected with BVDV NADL strain to allow virus replication. The supernatant containing virus particles was recovered, and the RNA of BVDV NADL was extracted using Ultrastructure RNA Kit (Shanghai Jing Biotechnology, Shanghai, China) following manufacturer's instructions. One-step real-time quantitative polymerase chain reaction (RT-qPCR) was employed to establish a standard curve of BVDV NADL strain and detect the BVDV RNA levels in each experimental group. The reaction mixture was prepared using OneStepPrimeScript™ RT-PCR Kit (Baobioengineering Dalian Co., Ltd., Dalian, Liaoning, China) with 10 μL of 2× One Step RT-PCR Buffer III, 0.5 μL of each probe, TaKaRa Ex Tap HS (5 U/μL), reverse and forward primers, and PrimeScript RT Enzyme Mix II, 3 μL of total RNA, 4.5 μL of RNase free dH_2O. The reaction was performed using HM-P16 Fluence Quantitative PCR Instrument (Shandong Hengmei Electronic Technology Co., Ltd., Weifang, Shandong, China). The content of virus was calculated according to the established standard curve and Ct values of each group.

The effect of baicalin on autophagy gene expression

Table 1. The sequences of BVDV primers and probes.

Primer and probe name	Sequence
BVDV-forward	5'- GGA TGC CAT GTG GAC GAG GGC G -3'
BVDV-reverse	5'- GCA TGT GCC ATG TAC AGC AGA GG -3'
Probes	FAM- CAA TAC AGT GGG CCT CTG CAG CA -TAMRA

Table 2. Autophagy gene primer sequences.

Primer name	Sequence	GenBank ID
GAPDH-F	5'- TTG TGA TGG GCG TGA ACC -3'	AF239959
GAPDH-R	5'- CCC TCC ACG ATG CCA AA -3'	
Atg12-F	5'- GGG GAT GAA CTA CAG AGG AAA AGA -3'	NM-001076982.1
Atg12-R	5'- GAA CAG ATT ACA ACC ACA AGA CGA A -3'	
Beclin-1-F	5'- TGA GAC TCC GGG TCA GAA TG -3'	NM-001034522.2
Beclin-1-R	5'- ACA CGT ACA GTT CAA GAT TCC ACA -3'	
LC3B-F	5'- GTT TCA AGT TAG CAC TCT CGT TCC A -3'	NM-001001169.1
LC3B-R	5'- TTT ACC AAA GCA GGC ACC ATT C -3'	

After treating cells with baicalin, the total RNAs of each experimental group were extracted at 12, 24, and 48 of cultivation. The reverse transcription was performed using RT Primer Mix and PrimeScript™ with 1 μL of RT Enzyme Mix I, 1 μL of 5× PrimeScript™, 4 μL of Buffer 2, 4 μL of RNase free dH_2O, and 10 μL of Degenome reaction solution at 37℃ for 15 mins and 85℃ for 5 s. The obtained cDNA was stored at 20℃ for subsequent fluorescence quantitative PCR (qPCR) experiment. The primers of Atg-12, Beclin-1, LC3B autophagy genes and internal reference gene GAPDH were designed using Primer 6.0 and synthesized by Shenggong Biotechnology Co., Ltd. (Shanghai, China) (Table 2). The qPCR reaction consisted of 1 μL each of forward and reverse primers, 2 μL of cDNA, 6 μL of ddH_2O, and 10 μL of SYBR® Premix Ex Taq™ II. The reaction was performed at 95℃ for 5s, followed by 40 cycles of 56℃ for 30 s. The Ct values of each group were recorded, and the relative expression levels of related autophagy genes were analyzed.

The effect of baicalin on BVDV induced cell apoptosis.

After cell treatments, the cell samples were taken at 96 h to detect the level of cell apoptosis using Attune NxT Flow cytometry (ThermoFisher Scientific, Shanghai, China). Briefly, the cells were washed 3 times with PBS before drying and digesting with 0.25% trypsin. The cells were then collected and centrifuged at 1,000 for 5 mins. After discarding the supernatant and resuspending with PBS, cells were centrifuged again for 5 mins. The binding buffer was added and resuspended the cells. After performing cell counting, the cell number was adjusted to 106 cells per tube and transferred to flow cytometry tube with 100 μL/tube. 5 μL of Annexin V-FITC and an equal volume of PI working solution were added to each cell tube. After incubating on ice for 15 mins, the sample was loaded on flow cytometry to determine cell apoptosis in each group.

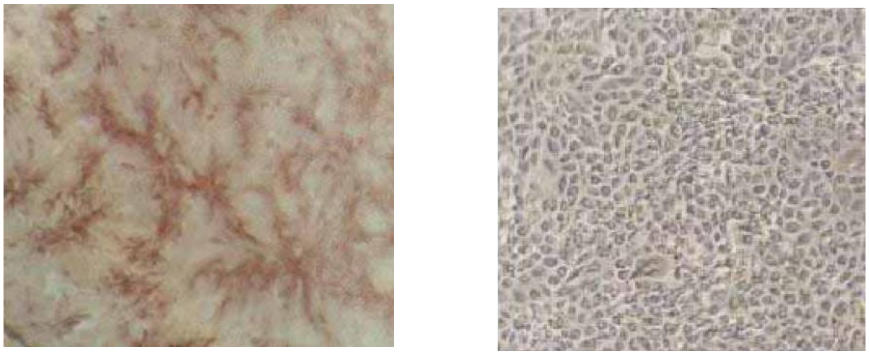
Statistical analysis

SPSS 23.0 (IBM, Armonk, New York, USA) was employed for the statistical analysis of this study. Student t-test was applied for inter group comparison with *P* value less than 0.05 as the statistically significant difference.

Results and discussion

Determination of TCID50

The antiviral pathways of Traditional Chinese Medicine are mainly divided into direct and



(a) BVDV (b) Control group

Figure 1. BVDV TCID50 measurement results.

indirect two types. The direct antiviral effect mainly refers to the direct killing of the virus, as well as the blocking of the virus's adsorption, penetration, replication, and other processes on normal cells to achieve the purpose of antiviral treatment [22]. The indirect antiviral effect refers to the inhibition of virus replicate in the body cells after infection. Therefore, by enhancing and stimulating the body's immune system, it can indirectly exert antiviral effects. The TCID50 was obtained through the comparison of BVDV control group (VCG) that cells were infected by BVDV only and normal cell group (NCG) (Figure 1). The cells infected with BVDV demonstrated blurred boundaries and exhibited pathological changes, which were stained red brown (Figure 1a), while the normal cell control group showed normal cell growth, clear cell boundaries, no structural damage, no pathological changes, and no staining (Figure 1b). The results indicated that BVDV could damage the structure of cells and cause pathological changes.

The anti-BVDV activity of baicalin

The inhibitory effect of baicalin on BVDV invasion was shown in Figure 2. As the concentrations of baicalin gradually increased, the OD_{450} values also gradually increased. When the concentration of baicalin reached 12.5 μg/mL, the OD_{450} value was 0.714 (Figure 2a). The cell survival rate increased with the increase of baicalin concentrations. When the concentration of baicalin increased from 0.049 to 12.5 μg/mL, the cell survival rate also increased from 56.85 to 89.06% (Figure 2b). The antiviral efficacy of baicalin also increased with the increase of baicalin concentrations. When the concentration of baicalin was 12.5 μg/mL, the antiviral effective rate of baicalin reached 85.83%. The results demonstrated that baicalin had a good invasion blocking effect on BVDV, and the higher the concentration of baicalin, the better the invasion blocking effect on BVDV.

Determination of baicalin's IC_{50}

A comparative experiment was conducted between the baicalin drug group (BDG) that treated MDBK cells with different baicalin concentrations and the normal cell control group (NCG). After 12 hours of baicalin administration, the safe concentration of baicalin to the MDBK cells was determined using the CCK-8 method. The cells in 100 μg/mL BDG demonstrated a toxic reaction (Figure 3a), while the cells of NCG were still normal (Figure 3b). The IC_{50} of baicalin was then determined as 45.31 μg/mL (Figure 3c). The dose-response regression equation of baicalin on MDBK cells is listed below.

$$y = 0.5828x - 0.465$$

Baicalin inhibited BVDV RNA copy numbers

The probe method was applied to detect the BVDV RNA contents of each experimental group with the standard curve established with the logarithm of standard RNA dilution (x) and the Ct

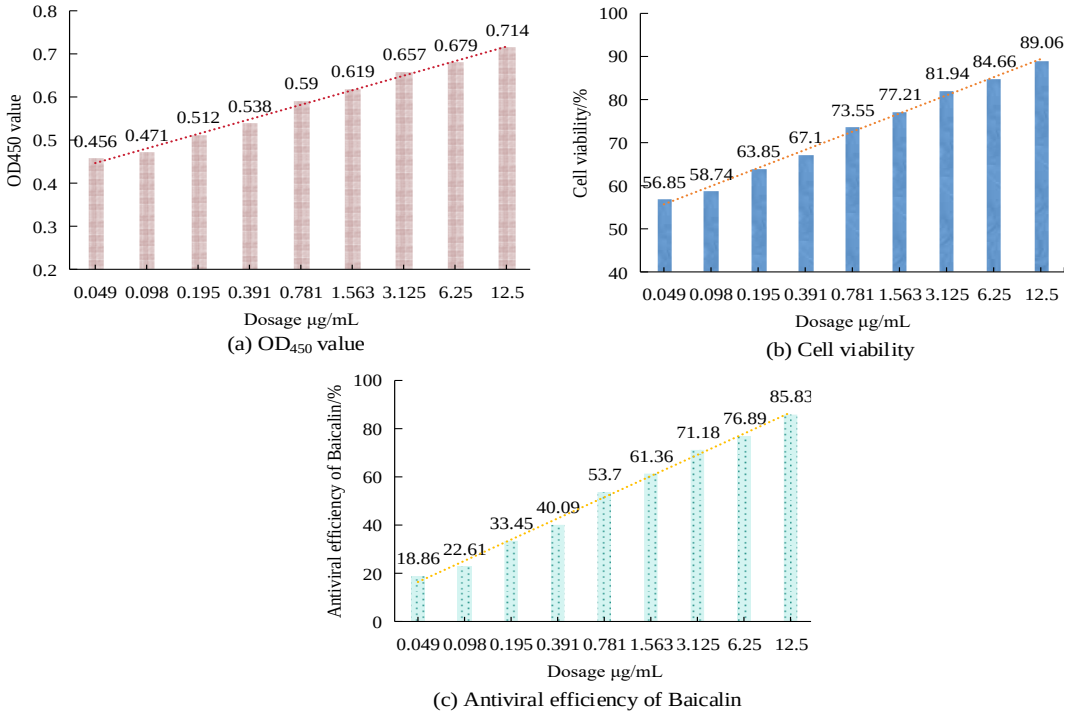


Figure 2. Invasion blocking effect of baicalin on BVDV.

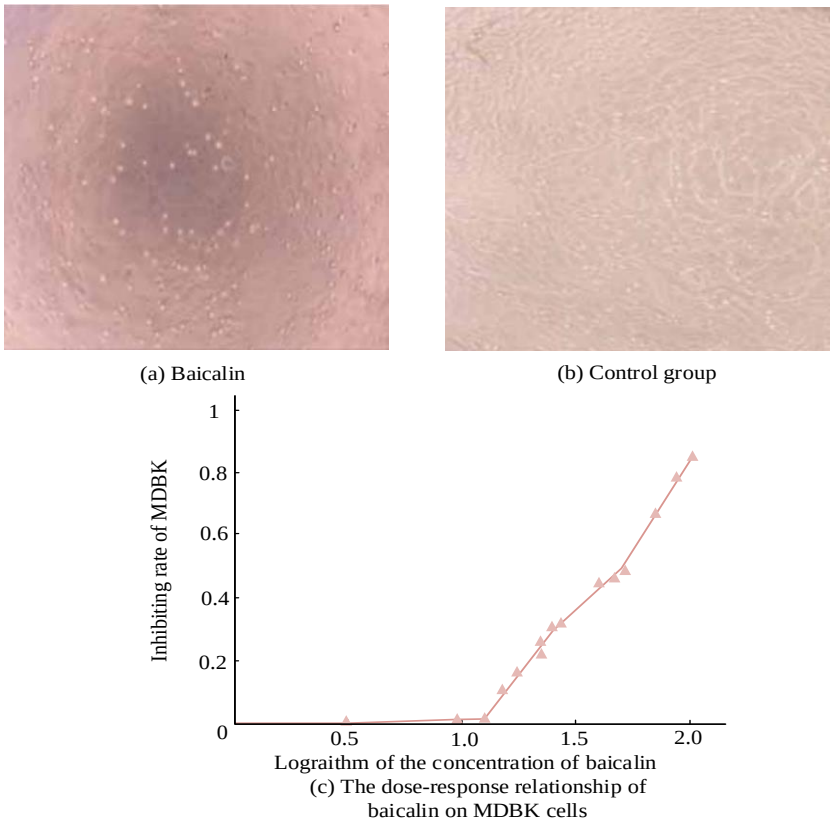


Figure 3. The dose-response relationship between baicalin toxicity to MDBK cells and its effect on MDBK cells.

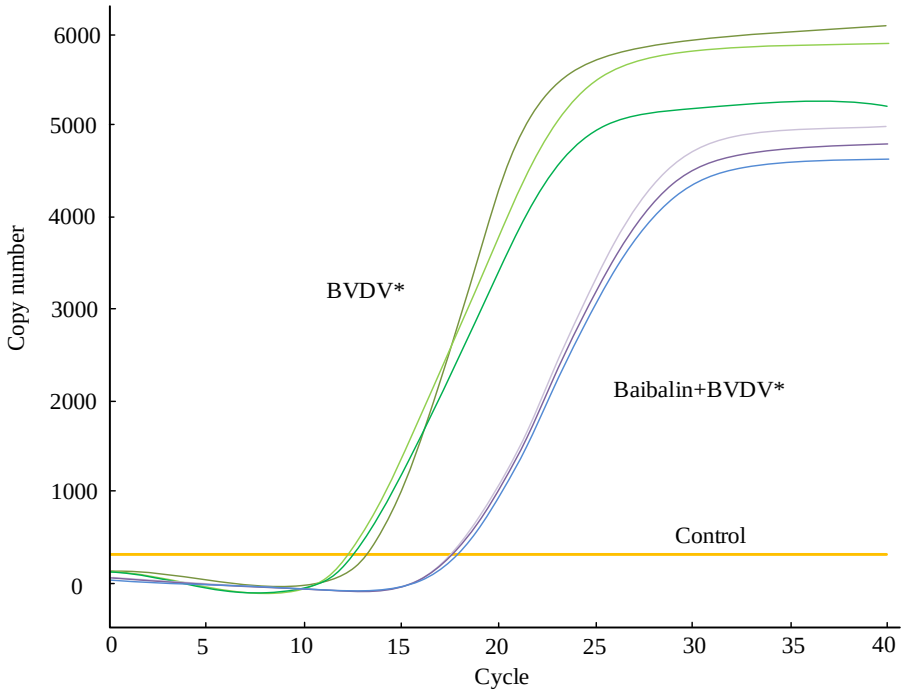


Figure 4. BVDV RNA copy numbers between BVDV group and baicalin + BVDV group. *: $P < 0.05$, indicating a significant difference between the two groups.

value (y) in equation below.

$$y = -3.027x + 38.03$$

The BVDV RNA copy numbers in the control, BVDV only, and baicalin + BVDV groups were 0, $10^{8.188}$/μL, and $10^{6.38}$/μL, respectively (Figure 4). The BVDV RNA copy number in baicalin + BVDV group was significantly lower than that in the BVDV group ($P < 0.05$), which indicated that baicalin could effectively inhibit the replication of virus inside the cells and had a good BVDV inhibitory effect.

The expression levels of autophagy related genes

The expression levels of autophagy related genes including *Atg-12*, *Beclin-1*, and *LC3B* genes were inspected in the groups of control, BVDV, and baicalin + BVDV at 12, 24, and 48 h after BVDV infection. The mRNA levels of the autophagy gene Beclin-1 gradually increased over the time in all three groups with the BVDV group remaining the highest one and reaching the peak at 48 h, which was significantly higher than that in the baicalin + BVDV group ($P < 0.05$) (Figure 5a). The mRNA level of Atg-12 in the BVDV group was consistently the highest and reached 2.43 at 48 h (Figure 5b). However, it was not significantly different from those in the baicalin + BVDV group at 12 h after infection ($P > 0.05$), but it showed the significantly difference to the baicalin + BVDV group at 24 and 48 hours ($P < 0.05$). The mRNA level of LC3B in the BVDV group was also consistently the highest and reached 2.58 at 48 h (Figure 5c). The results indicated that baicalin could inhibit the mRNA level of autophagy genes by inhibiting the replication of BVDV.

Baicalin effect on cell apoptosis

After 96 h of cell culture, flow cytometry was used to detect apoptosis in the three groups. The results showed that the BVDV group had the highest cell apoptosis followed by the control group. The baicalin + BVDV group demonstrated the least cell apoptosis (Figure 6). The result suggested that baicalin could significantly reduce cell apoptosis caused by BVDV.

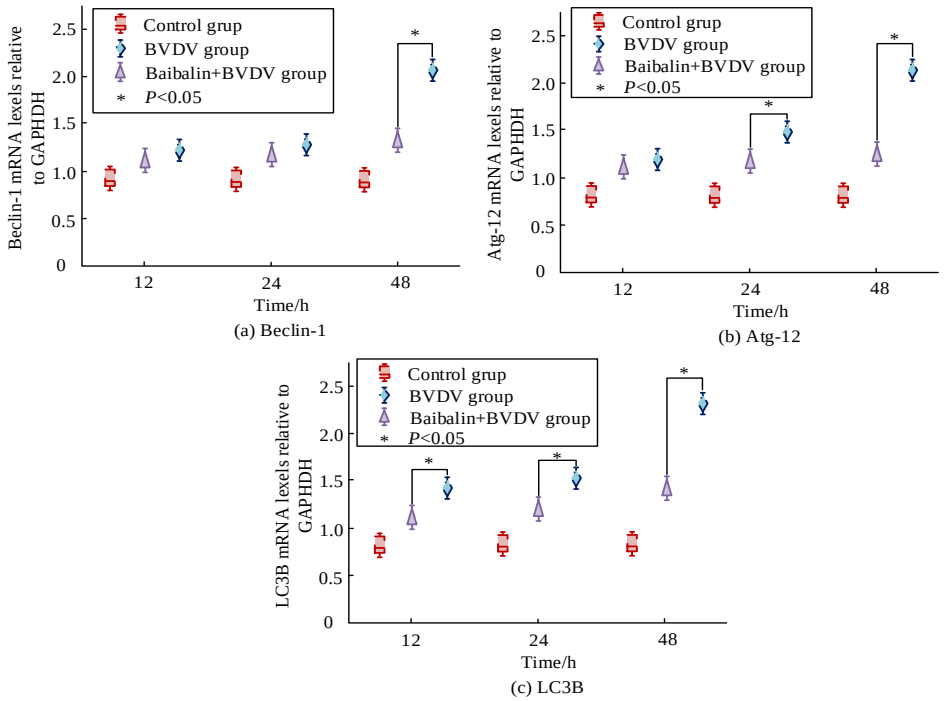


Figure 5. Autophagy gene expression level.

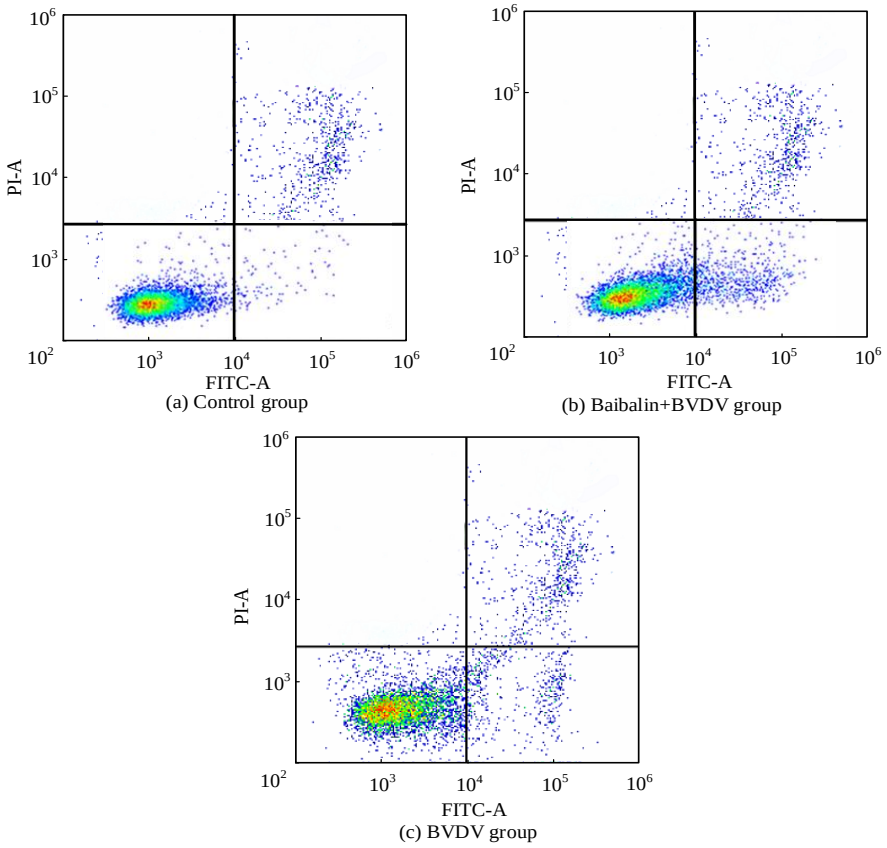


Figure 6. Cell apoptosis situation.

Conclusion

With the continuous increase in consumer demand for agricultural products, animal husbandry has ushered in rapid development. However, how to keep animal husbandry in a healthy and sustainable development trend is still an urgent problem. To explore the feasibility of using baicalin as a potential BVDV antiviral drug, this study analyzed the activity and mechanism of baicalin against BVDV. The results indicated that BVDV could damage the structure of cells and cause pathological changes. Baicalin had a good invasion blocking effect on BVDV with the trend of the higher the concentration, the better the invasion blocking effect. Baicalin could effectively inhibit the replication of virus and had a good BVDV inhibitory effect. Baicalin could inhibit the mRNA levels of autophagy genes by inhibiting the replication of BVDV. In addition, baicalin could significantly reduce cell apoptosis caused by BVDV. However, the research on the antiviral effect of baicalin alone on BVDV was still limited. Therefore, in future research, the feasibility of combining baicalin with other methods to combat BVDV should be further explored to improve the antiviral effect on BVDV and promote the healthy development of animal husbandry.

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